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GENE TRANSFER PREPARATION

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GENE TRANSFER PREPARATION

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Technical field

This invention pertains to a method for manufacturing safe lyophilized preparations of virus vectors for gene therapy with excellent storage stability and gene transfer preparations obtained with said method.

Prior art

With the rapid development of genetic engineering, various molecular biological techniques have been developed. Along with this, remarkable progress has been seen in analysis of genetic information and elucidation of gene function, and many attempts are being made to use the fruits obtained from these in actual treatment settings. Among these, the field of gene therapy can be cited as one of the areas in which progress is most conspicuous. While causative genes in various genetic diseases are being discovered and deciphered, methods for introducing these genes into cells by physical and chemical techniques are being developed, and gene therapy has progressed from the stage of basic experiments to actual clinical applications.

For clinical applications of gene therapy, since the first clinical tests of gene therapy were conducted in the U.S. in 1989, clinical tests have also been started in Italy, Holland, France, England and China. In the U.S. in particular, 54 gene therapy protocols had been approved by the Recombinant DNA committee (RAC) of NIH by July 1994. Gene therapies are being tried for genetic diseases such as congenital immunodeficiency (adenosine deaminase deficiency), familial hypercholesterolemia, and cystic fibrosis, and various cancers such as gliomas and malignant melanoma. Recently, many basic studies on gene therapy for AIDS are also being conducted.

Gene therapies are classified as Germline Cell Gene Therapy or Somatic Cell Gene Therapy by the variety of cell (target cell) into which the gene is introduced. They are also classified as Augmentation Gene Therapy, wherein the abnormal (causative) gene is left as is and a new (normal) gene is added, or Replacement Gene Therapy, wherein the abnormal gene is replaced with a normal gene. But at present, due to ethical and technical limitations, only augmentation gene therapy in somatic cells is being performed. Furthermore, for gene therapy methods, autotransplantation methods (ex vivo gene therapy) are being performed at present wherein target cells are taken out of patients' bodies and those cells are returned to the patients' bodies after introducing the desired genes. Methods for administering genes directly to the patient in the future (in vivo gene therapy) are also being studied.

One important technical topic in clinical applications of gene therapy like the above is how to introduce exogenous genes efficiently and safely into target cells. In the early 1980s, adaptations of physical techniques such as microinjection were tried. But gene transfer efficiency was low and stable transfer was not possible. There were also limits on large volume cell

culturing techniques at the time, and these methods did not lead to practical use. Subsequently, recombinant viruses (virus vectors) that would serve as vectors for introducing exogenous genes efficiently into target cells were developed, making clinical applications of gene therapy possible for the first time.

There are several varieties of virus vectors as given below. The virus vector most widely used in gene therapies that are presently being performed is a retrovirus vector derived from mouse leukemia virus (MoMLV: Moloney Murine Leukemia Virus). It takes advantage of the proliferation mode of this virus. Retroviruses are RNA viruses having envelopes, and invade cells by these envelope proteins binding to receptors on the host cell. After invasion, the single-stranded virus RNA is converted to double-stranded DNA by reverse transcriptase and is incorporated randomly but stably into the genome DNA of the infected cell. In order to be incorporated, the cell must be dividing and multiplying (D.G. Miller, et al., Molecular and Cellular Biology, 10, 8, 4239, 1990). The incorporated retrovirus gene is called a provirus. From this provirus, RNA is transcribed and viral protein is synthesized. New virus particles are formed by these proteins and viral RNA. Retrovirus vectors are ones in which the retrovirus gene here has been recombined with an exogenous gene (A.D. Miller, Current Topics in Microbiology and Immunology, 158, 1, 1992). There has already been a very large number of studies on retrovirus vectors, particularly MoMLV vectors, and many improvements on their safety have been made. No significant problems have occurred to date. However, MoMLV vectors are known to have the properties that incorporation into target cell genome DNA is random and that the long terminal repeat (LTR below), which is a part of the viral gene, has gene expression-promoting activity. For this reason, although there have been no previous reports, the possibility that, as a result of random incorporation of an exogenous gene, a cancer gene that happens to be nearby is activated and makes the target cell cancerous, cannot be completely dismissed. The development of a safer vector was strongly desired. Furthermore, what was the biggest problem with MoMLV vectors in practical use was the fact that genes could not be transferred to non-dividing cells. For this reason, restoration of genes in nerve cells, which is a problem in many congenital metabolic abnormalities, could not be performed. In addition to this, because hematopoietic stem cells, liver cells, muscle cells, etc., that are target cells for gene therapy also are normally in an essentially quiescent phase, gene transfer efficiency is low. For cells that have been removed from the body, treatments to promote division are performed to increase gene transfer efficiency. But it is thought that transferring genes to these cells in the body would be difficult and it is said that development of vectors that can transfer genes efficiently even to non-dividing cells will be necessary in the future.

Herpes virus vectors are anticipated as vectors capable of transferring exogenous genes to nerve cells (T.D. Palella, et al., Mol. Cell. Biol., 8, 457, 1988). But because cytotoxicity is strong

and the genome size of the virus itself is a very large 150 kb, development is not progressing at present.

HIV vectors were developed as vectors capable of specific gene transfer to CD4-positive T-lymphocytes due to the host characteristics of the virus itself (T. Shimada, et al., J. Clin. Invest., 88, 1043, 1991). Because lymphocytes are important target cells when performing gene therapy for congenital immunodeficiency, AIDS, cancers, etc., high expectations are placed on the usefulness of HIV vectors. As the most significant drawback of HIV vectors, there is the problem of possible intermixing of wild strains. But if this can be resolved, it may be possible to use these for in vivo gene therapy by intravascular administration.

Adenovirus vectors have been drawing the most attention recently because they can also transfer genes to non-dividing cells and the vectors can easily be concentrated to about the 10^{th} power of 10. From recent research, it has been shown that genes can be transferred efficiently in vivo to airway epithelial cells, liver cells, muscle cells, etc., with these adenovirus vectors (L.D. Lavrero, et al., Hum. Gene Therapy, 1, 241, 1990, B. Quantin, Proc. Natl. Acad. Sci. U.S.A., 89, 2581, 1992). On the other hand, these vectors have an intrinsic property that when they transfer exogenous genes into the cell, they are not incorporated into genome DNA. If vectors are allowed to act on target cells, the effects of gene transfer disappear in several weeks or at most, several months. For this reason, gene transfer must be repeated frequently. Increased physical and psychological burden on the patient and reduction in gene transfer efficiency due to appearance of anti-adenovirus antibodies are problems. Moreover, clinical trials of administering adenovirus vectors to the lungs bronchoscopically for treatment of cystic fibrosis have begun. It is said that inflammatory reactions occur that are thought to arise from the immunogenicity and cytotoxicity of the adenovirus particles.

Meanwhile, AAV (adeno-associated virus) vectors have characteristics such as exogenous genes being incorporated into target cell genome DNA and their not having pathogenicity or cytotoxicity (N. Muzyczka, Current Topics in Microbiology and Immunology, 158, 97, 1992). Moreover, from the fact that the ITR (inverted terminal repeat), which is necessary for packaging into virus particles and for incorporation of genes into genome DNA, does not have gene expression-promoting activity, by establishing an internal promoter that suits the objective, on/off of gene expression and use of tissue-specific promoters become possible. Because the host range is also broad and they can deal with various target cells/diseases, they are anticipated as novel virus vectors that will replace MoMLV vectors. It has also been discovered that wild type AAVs are incorporated into a specific position on the No. 19 chromosome (M. Suwadogo and R.G. Roeder, Proc. Natl. Acad. Sci. U.S.A., 82, 4394, 1985) and they are drawing attention as vectors for which gene incorporation position can be targeted.

However, for the virus vectors, no pharmaceutical studies have been conducted for their stable storage and maintenance of homogeneity. At present, virus vectors are stored frozen and then used. But there are limits on storage time, and virus vector potency has been observed to drop with passage of time. For this reason, it is necessary to manufacture vectors for each trial in actual clinical studies and, at the same time, to test, prior to treatment, for decreases in gene transfer efficiency during storage. Because methods are extremely complex and considerable amounts of time are also necessary for results to be obtained for such tests, the establishment of a method for supplying stabilized virus vectors with uniform performance is strongly desired.

There have been attempts to lyophilize MoMLV vectors (N. Kotani et al., Human Gene Therapy, 5, 19, 1994). But gelatin was used as stabilizing agent. For gelatin, normally products derived from animals such as pigs are used, and for this reason, the possibility of becoming an immunogen when these are administered to the body in vivo is high and it cannot necessarily be called a safe method.

In clinical studies of gene therapy being conducted at present, varieties of vectors used and pharmacological effects of therapeutic genes are being studied in detail. However, since virus vectors are preparations for gene therapy, given that more gene therapies will be conducted in the future, development of technology to supply safe vectors with uniform properties stably, that is, methods for stable storage of vectors, is necessary and indispensable. However, there are very few studies pertaining to such fields.

Presentation of the invention

Upon diligent research to solve the problems mentioned above, the inventors succeeded in developing a technology for supplying safe virus vectors of uniform properties stably, that is, a method for stable storage of virus vectors. This makes it possible to lyophilize various kinds of virus vectors without reducing gene transfer efficiency.

In the past, it has been known that several varieties of viruses do not lose infectivity even when lyophilized. When doing so, the addition of gelatin and sugars as additives was common. Meanwhile, attempts at lyophilization were made with virus vectors that are similar to viruses, as mentioned above. However, gelatin and sugars were added as additives in these cases as well. The possibility of becoming immunogens when given to the body in vivo was high and it could not necessarily be called a safe method.

Therefore, in this invention, a lyophilization method was developed that kept gene transfer efficiency high without including components such as gelatin that could become immunogens and that used, as additives, only low molecular weight substances for which there were precedents for use as medicinal additives.

That is, this invention presents a method for manufacturing gene transfer preparations comprising the addition of 1 or more additives selected from arginine, glutamic acid (or its sodium salt), serine, glucose, inositol, lactose, mannitol, sorbitol, trehalose or xylose to recombinant virus vectors, and lyophilization.

This invention also presents gene transfer preparations manufactured by the above method.

Brief description of the figures

Figure 1 is a graph showing the efficiency of gene transfer when various kinds of additives were added at a concentration of 5%.

Figure 2 is a graph showing gene transfer efficiency when various kinds of additives were added at 2.5% and 5% concentrations.

Figure 3 is a graph showing gene transfer efficiency when 2 kinds of additives of 2.5% concentration were combined and added.

Optimal embodiment of the invention

Virus vectors for which the method of this invention can be used can be any virus vector that is used in gene therapy, such as the MoMLV (murine leukemia virus) vector mentioned above, Herpes virus vectors, adenovirus vectors, adeno-associated virus (AAV) vectors, human immunodeficiency virus (HIV) vectors, etc. Such virus vectors are solubilized in media such as DMEM medium or PBS and a virus vector stock solution is prepared. The virus vector stock solution can be any concentration.

The method of this invention is achieved by adding additives to the above virus vector stock solution and lyophilizing this. Substances of low immunogenicity are preferable for the additives that can be used in this invention, and they include low molecular weight amino acids and their derivatives, and sugars and their derivatives.

For amino acids and their derivatives, arginine, glutamic acid (or its sodium salt) and serine are favorable. Glutamic acid and its sodium salt are particularly favorable.

For sugars and their derivatives, glucose, inositol, lactose, mannitol, sorbitol, trehalose and xylose are favorable. Glucose is most favorable.

Depending on the variety of virus vector used and concentration of virus vector solution, 1 or more selected from these amino acids and sugars can be combined freely and used as additives. One can select from combinations of amino acids only, combinations of sugars only and combinations of amino acids with sugars. Combinations of sodium glutamate with glucose are most favorable from the standpoint of maintaining high gene transfer efficiency (virus vector potency).

The weight ratio of each additive selected from amino acids and sugars with respect to vector solution is about 1-about 10% each, preferably about 1.5-about 7% each and most preferably about 1.5-about 5%.

Ascorbic acid, polyethyleneglycol, polyvinylpyrrolidone, polyvinyl alcohol and preservatives can also be added. It is also preferable that the virus vector solution is isotonic. For this reason, buffers can be added to the virus vector solution and the osmotic pressure adjusted.

Virus vector solutions thus obtained with various additives added are lyophilized. For the lyophilization, well-known methods can be used. For example, after freezing with liquid nitrogen, it can be performed by a lyophilizer (made by Finaqua Co.). Lyophilized gene transfer preparations are sealed in vials and stored until use, preferably at low temperature. The gene transfer preparations of this invention can be regenerated with water at time of use. As shown in the application examples below, virus vectors regenerated with water had retained high gene transfer efficiency.

With this invention, gene transfer preparations can be obtained that maintain high gene transfer efficiencies without including components such as gelatin that could become immunogens and using only low molecular weight substances for which there already are precedents for use as additives in medicinal products. For the gene transfer preparations of this invention, storage is easy, potency can be maintained stably, the method can be used for various virus vector preparations, and its range of application is very wide.

Application examples

Application examples are given below and this invention is explained in further detail. But this invention is not limited by the following examples.

Application Example 1: Preparation of recombinant MoMLV vectors

On 9-cm diameter cell culturing dishes, PA317/β-19 (provided by Professor Shimada, Nippon Medical School), which is a cell producing recombinant MoMLV vectors that include neomycin resistance gene, was sown and cultured in DMEM (made by Gibco Co.) containing 10% fetal calf serum (made by Gibco Co.) to 80% confluence under the usual conditions (37°C, 5% carbon dioxide concentration). After culturing the cells to 80% confluence, medium was exchanged. 12 hours later, medium containing recombinant MoMLV vectors was collected and this was used as the vector stock solution.

Application Example 2: Preparation of recombinant MoMLV vector solution and lyophilization

To the vector stock solution obtained in Application Example 1, the amino acids, sugars or combinations thereof recorded in Figures 1-3 were added as additives to give final

concentrations of 5% or 2.5%. After freezing with liquid nitrogen, lyophilization was performed for one whole day and night with a lyophilizer (made by Finaqua Co.). The lyophilized products were stored at -40°C until use. Products to which additives were not added were also prepared and these were used as controls. All of the additives were made by Wako Jun'yaku Co.

Application Example 3: Method for measuring recombinant MoMLV vector potency (gene transfer efficiency)

In cell culturing dishes of 6 cm diameter, 3T3 cells (made by Dainippon Pharmaceutical Co.) were sown and cultured in DMEM (made by Gibco Co.) containing 10% fetal calf serum (made by Gibco Co.) to 80% confluence under the usual conditions (37°C, 5% carbon dioxide concentration). After culturing the cells to 80% confluence, they were used for potency measurements.

To the lyophilized products obtained in Application Example 2, injection-quality distilled water (made by Otsuka Seiyaku Co.) was added and resuspended vector suspensions were prepared to the same volumes as before lyophilization. 10 μL of the obtained vector resuspensions and 990 μL of DMEM containing 10% fetal calf serum were added together and mixed and potency measurement vector solutions were prepared. The culture medium of the 3T3 cells cultured to 80% confluence was removed. 1000 μL of vector solutions for potency measurement were added thereto and culturing was performed under the usual conditions. 4 hours later, 3 mL of DMEM containing 10% fetal calf serum were added and culturing was continued for another 24 hours. After this, 10% fetal calf serum-containing DMEM containing 800 μg/mL G418 (made by Gibco Co.), a neomycin analog, was added and culturing was continued. The number of drug-resistant colonies formed was used to determine potency (cfu/mL).

Application Example 4: Potency measurements of recombinant MoMLV vectors

The results when 5% concentrations of various additives were used are shown in Figure 1. It was found that potency was high with glucose, sodium glutamate, mannitol, and trehalose. The results when 2.5% of these were added and when 2 kinds of additives were mixed and used are shown in Figures 2 and 3. From the above results, it was found that by using glucose and sodium glutamate as additives, lyophilized products of virus vectors having high potency could be obtained.

Claims

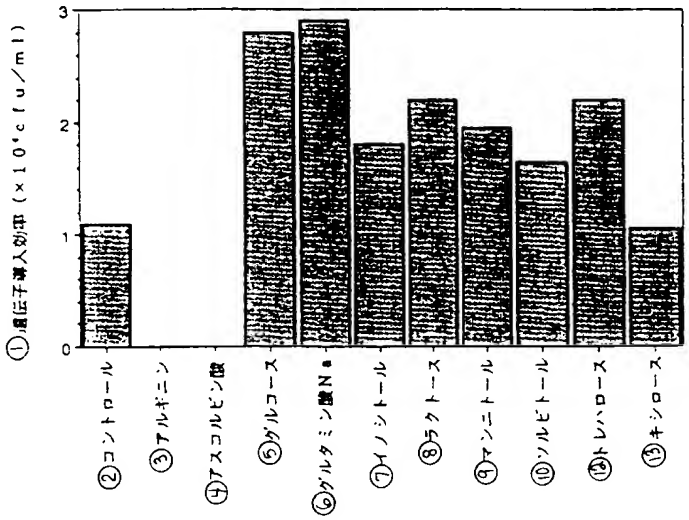
1. Method for manufacturing gene transfer preparations comprising the addition of 1 or more additives selected from arginine, glutamic acid (or its sodium salt), serine, glucose, inositol,

lactose, mannitol, sorbitol, trehalose and xylose to recombinant virus vectors, followed by lyophilization.

2. Method described as in Claim 1, wherein the weight ratio of various additives with respect to vector solution is about 1-10% each.

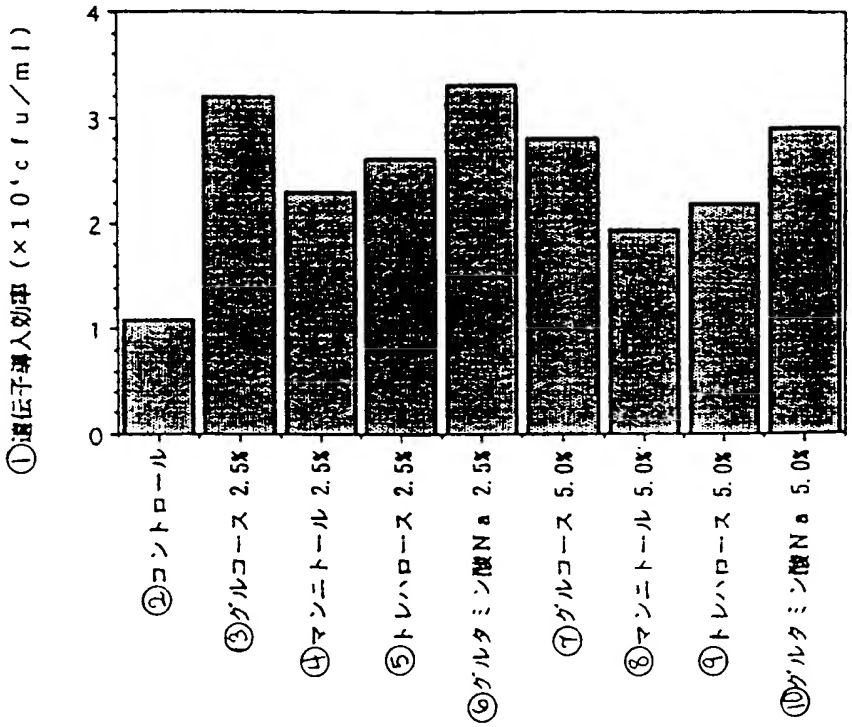
3. Method described as in Claim 1, wherein the additive is a combination of glutamic acid (or its sodium salt) with glucose.

4. Gene transfer preparations manufactured by the method of Claim 1.

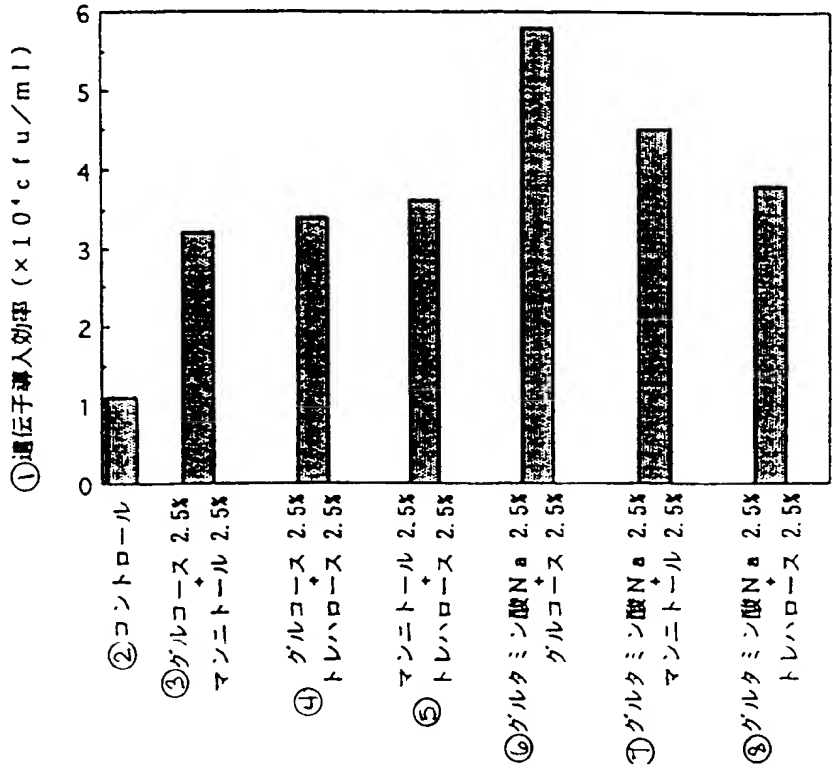


Key:
1 Gene transfer efficiency (x 10^4 cfu/mL)
2 Control
3 Arginine
4 Ascorbic acid
5 Glucose
6 Na glutamate
7 Inositol
8 Lactose
9 Mannitol
10 Sorbitol

11 Trehalose
12 Xylose



Key: 1 Gene transfer efficiency (x 10^4 cfu/mL)
2 Control
3 Glucose 2.5%
4 Mannitol 2.5%
5 Trehalose 2.5%
6 Na glutamate 2.5%
7 Glucose 5.0%
8 Mannitol 5.0%
9 Trehalose 5.0%
10 Na glutamate 5.0%



Key:
1 Gene transfer efficiency (x 10^4 cfu/mL)
2 Control
3 Glucose 2.5%
Mannitol 2.5%
4 Glucose 2.5%
Trehalose 2.5%
5 Mannitol 2.5%
Trehalose 2.5%
6 Na glutamate 2.5%
Glucose 2.5%
7 Na glutamate 2.5%
Mannitol 2.5%

8 Na glutamate 2.5%
Trehalose 2.5%

INTERNATIONAL SEARCH REPORT

International application No.
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A. CLASSIFICATION OF SUBJECT MATTER
Int. Cl[6] A61K48/00, 9/14, 31/70, 35/76, 47/10, 47/18, 47/26 //
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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	By Tomonokai of National Institute of Health "Rev. ed. 2 Experimental Virology General Description" (1973), Maruzen, p. 53-59, Section of "5·2 Freeze-drying"	1 - 4
A	WO, 95/19427, A (Genetic Therapy Inc.), July 20, 1995 (20. 07. 95)(Family: none)	1 - 4
A	Chemical Abstracts, Vol. 121 (1994) Abstract No. 223170 (Human Gene Therapy, Vol. 5 No. 1 (1994), p. 19-28, (H. Kotani et al.),	1 - 4

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
June 6, 1996 (06. 06. 96)	June 18, 1996 (18. 06. 96)
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